

1                    **INDIVIDUALIZED ANTI-CANCER ANTIBODIES**

2        **Reference to Related Applications:**

3            This application is a continuation of application S.N.  
4        09/415,278, filed October 8, 1999, now U.S. Patent  
5        \_\_\_\_\_, the contents of which are herein incorporated  
6        by reference.

7        **Field of the Invention:**

8            This invention relates to the production of anti-cancer  
9        antibodies customized for the individual patient that can be  
10       used for therapeutic and diagnostic purposes. The invention  
11       further relates to the process by which the antibodies are  
12       made and to their methods of use.

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14        **Background of the Invention:**

15           Each individual who presents with cancer is unique and  
16       has a cancer that is as different from other cancers as that  
17       person's identity. Despite this, current therapy treats all  
18       patients with the same type of cancer, at the same stage, in  
19       the same way. At least 30% of these patients will fail the  
20       first line therapy, thus leading to further rounds of  
21       treatment and the increased probability of treatment failure,  
22       metastases, and ultimately, death. A superior approach to  
23       treatment would be the customization of therapy for the  
24       particular individual. The only current therapy which lends

1 itself to customization is surgery. Chemotherapy and  
2 radiation treatment can not be tailored to the patient, and  
3 surgery by itself, in most cases is inadequate for producing  
4 cures.

5 With the advent of monoclonal antibodies, the  
6 possibility of developing methods for customized therapy  
7 became more realistic since each antibody can be directed to  
8 a single epitope. Furthermore, it is possible to produce a  
9 combination of antibodies that are directed to the  
10 constellation of epitopes that uniquely define a particular  
11 individual's tumor.

12 Having recognized that a significant difference between  
13 cancerous and normal cells is that cancerous cells contain  
14 antigens that are specific to transformed cells, the  
15 scientific community has long held that monoclonal antibodies  
16 can be designed to specifically target transformed cells by  
17 binding specifically to these cancer antigens; thus giving  
18 rise to the belief that monoclonal antibodies can serve as  
19 "Magic Bullets" to eliminate cancer cells.

20 At the present time, however, the cancer patient usually  
21 has few options of treatment. The regimented approach to  
22 cancer therapy has produced improvements in global survival  
23 and morbidity rates. However, to the particular individual,  
24 these improved statistics do not necessarily correlate with  
25 an improvement in their personal situation.

1           Thus, if a methodology was put forth which enabled the  
2 practitioner to treat each tumor independently of other  
3 patients in the same cohort, this would permit the unique  
4 approach of tailoring therapy to just that one person. Such  
5 a course of therapy would, ideally, increase the rate of  
6 cures, and produce better outcomes, thereby satisfying a  
7 long-felt need.

8           Historically, the use of polyclonal antibodies has been  
9 used with limited success in the treatment of human cancers.  
10 Lymphomas and leukemias have been treated with human plasma,  
11 but there were few prolonged remission or responses.  
12 Furthermore, there was a lack of reproducibility and there  
13 was no additional benefit compared to chemotherapy. Solid  
14 tumors such as breast cancers, melanomas and renal cell  
15 carcinomas have also been treated with human blood,  
16 chimpanzee serum, human plasma and horse serum with  
17 correspondingly unpredictable and ineffective results.

18           There have been many clinical trials of monoclonal  
19 antibodies for solid tumors. In the 1980s there were at least  
20 four clinical trials for human breast cancer which produced  
21 only one responder from at least 47 patients using antibodies  
22 against specific antigens or based on tissue selectivity. It  
23 was not until 1998 that there was a successful clinical trial  
24 using a humanized anti-her 2 antibody in combination with  
25 Cisplatin. In this trial 37 patients were accessed for

1 responses of which about a quarter had a partial response  
2 rate and another half had minor or stable disease  
3 progression.

4 The clinical trials investigating colorectal cancer  
5 involve antibodies against both glycoprotein and glycolipid  
6 targets. Antibodies such as 17-1A, which has some  
7 specificity for adenocarcinomas, had undergone Phase 2  
8 clinical trials in over 60 patients with only one patient  
9 having a partial response. In other trials, use of 17-1A  
10 produced only one complete response and two minor responses  
11 among 52 patients in protocols using additional  
12 cyclophosphamide. Other trials involving 17-1A yielded  
13 results that were similar. The use of a humanized murine  
14 monoclonal antibody initially approved for imaging also did  
15 not produce tumor regression. To date there has not been an  
16 antibody that has been effective for colorectal cancer.  
17 Likewise there have been equally poor results for lung  
18 cancer, brain cancers, ovarian cancers, pancreatic cancer,  
19 prostate cancer, and stomach cancer. There has been some  
20 limited success in the use of anti-GD3 monoclonal antibody  
21 for melanoma. Thus, it can be seen that despite successful  
22 small animal studies that are a prerequisite for human  
23 clinical trials, the antibodies that have been tested have  
24 been for the most part ineffective.

25 **Prior Patents:**

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1 U.S. Patent No. 5,869,268 is drawn to a method for  
2 producing a human lymphocyte producing an antibody specific  
3 to a desired antigen, a method for producing a monoclonal  
4 antibody, as well as monoclonal antibodies produced by the  
5 method. The patent is particularly drawn to the production  
6 of an anti-HD human monoclonal antibody useful for the  
7 diagnosis and treatment of cancers.

8 U.S. Patent No. 5,869,045 relates to antibodies,  
9 antibody fragments, antibody conjugates and single chain  
10 immunotoxins reactive with human carcinoma cells. The  
11 mechanism by which these antibodies function is two-fold, in  
12 that the molecules are reactive with cell membrane antigens  
13 present on the surface of human carcinomas, and further in  
14 that the antibodies have the ability to internalize within  
15 the carcinoma cells, subsequent to binding, making them  
16 especially useful for forming antibody-drug and antibody-  
17 toxin conjugates. In their unmodified form the antibodies  
18 also manifest cytotoxic properties at specific  
19 concentrations.

20 U.S. Patent No. 5,780,033 discloses the use of  
21 autoantibodies for tumor therapy and prophylaxis. However,  
22 this antibody is an antinuclear autoantibody from an aged  
23 mammal. In this case, the autoantibody is said to be one type  
24 of natural antibody found in the immune system. Because the  
25 autoantibody comes from "an aged mammal", there is no

1 requirement that the autoantibody actually comes from the  
2 patient being treated. In addition the patent discloses  
3 natural and monoclonal antinuclear autoantibody from an aged  
4 mammal, and a hybridoma cell line producing a monoclonal  
5 antinuclear autoantibody.

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7 **Summary of the Invention:**

8 This application teaches a method for producing patient  
9 specific anti-cancer antibodies using a novel paradigm of  
10 screening. These antibodies can be made specifically for one  
11 tumor and thus make possible the customization of cancer  
12 therapy. Within the context of this application, anti-cancer  
13 antibodies having either cell-killing (cytotoxic) or cell-  
14 growth inhibiting (cytostatic) properties will hereafter be  
15 referred to as cytotoxic. These antibodies can be used in  
16 aid of staging and diagnosis of a cancer, and can be used to  
17 treat tumor metastases.

18 The prospect of individualized anti-cancer treatment  
19 will bring about a change in the way a patient is managed. A  
20 likely clinical scenario is that a tumor sample is obtained  
21 at the time of presentation, and banked. From this sample,  
22 the tumor can be typed from a panel of pre-existing anti-  
23 cancer antibodies. The patient will be conventionally staged  
24 but the available antibodies can be of use in further staging  
25 the patient. The patient can be treated immediately with the



1 existing antibodies, and a panel of antibodies specific to  
2 the tumor can be produced either using the methods outlined  
3 herein or through the use of phage display libraries in  
4 conjunction with the screening methods herein disclosed. All  
5 the antibodies generated will be added to the library of  
6 anti-cancer antibodies since there is a possibility that  
7 other tumors can bear some of the same epitopes as the one  
8 that is being treated.

9 In addition to anti-cancer antibodies, the patient can  
10 elect to receive the currently recommended therapies as part  
11 of a multi-modal regimen of treatment. The fact that the  
12 antibodies isolated via the present methodology are  
13 relatively non-toxic to non-cancerous cells allow  
14 combinations of antibodies at high doses to be used, either  
15 alone, or in conjunction with conventional therapy. The high  
16 therapeutic index will also permit re-treatment on a short  
17 time scale that should decrease the likelihood of emergence  
18 of treatment resistant cells.

19 If the patient is refractory to the initial course of  
20 therapy or metastases develop, the process of generating  
21 specific antibodies to the tumor can be repeated for re-  
22 treatment. Furthermore, the anti-cancer antibodies can be  
23 conjugated to red blood cells obtained from that patient and  
24 re-infused for treatment of metastases. There have been few  
25 effective treatments for metastatic cancer and metastases

usually portend a poor outcome resulting in death. However, metastatic cancers are usually well vascularized and the delivery of anti-cancer antibodies by red blood cells can have the effect of concentrating the antibodies at the site of the tumor. Even prior to metastases, most cancer cells are dependent on the host's blood supply for their survival and anti-cancer antibody conjugated red blood cells can be effective against *in situ* tumors, too. Alternatively, the antibodies may be conjugated to other hematogenous cells, e.g. lymphocytes, macrophages, monocytes, natural killer cells, etc.

There are five classes of antibodies and each is associated with a function that is conferred by its heavy chain. It is generally thought that cancer cell killing by naked antibodies are mediated either through antibody dependent cellular cytotoxicity or complement dependent cytotoxicity. For example murine IgM and IgG2a antibodies can activate human complement by binding the C-1 component of the complement system thereby activating the classical pathway of complement activation which can lead to tumor lysis. For human antibodies the most effective complement activating antibodies are generally IgM and IgG1. Murine antibodies of the IgG2a and IgG3 isotype are effective at recruiting cytotoxic cells that have Fc receptors which will lead to cell killing by monocytes, macrophages, granulocytes

1 and certain lymphocytes. Human antibodies of both the IgG1  
2 and IgG3 isotype mediate ADCC.

3 Another possible mechanism of antibody mediated cancer  
4 killing may be through the use of antibodies that function to  
5 catalyze the hydrolysis of various chemical bonds in the cell  
6 membrane and its associated glycoproteins or glycolipids, so-  
7 called catalytic antibodies.

8 There are two additional mechanisms of antibody mediated  
9 cancer cell killing which are more widely accepted. The  
10 first is the use of antibodies as a vaccine to induce the  
11 body to produce an immune response against the putative  
12 cancer antigen that resides on the tumor cell. The second is  
13 the use of antibodies to target growth receptors and  
14 interfere with their function or to down regulate that  
15 receptor so that effectively its function is lost.

16 Accordingly, it is an objective of the invention to  
17 teach a method for producing anti-cancer antibodies from  
18 cells derived from a particular individual which are  
19 cytotoxic with respect to cancer cells while simultaneously  
20 being relatively non-toxic to non-cancerous cells.

21 It is an additional objective of the invention to  
22 produce novel anti-cancer antibodies.

23 It is a further objective of the instant invention to  
24 produce anti-cancer antibodies whose cytotoxicity is mediated  
25 through antibody dependent cellular toxicity.

1 It is yet an additional objective of the instant  
2 invention to produce anti-cancer antibodies whose  
3 cytotoxicity is mediated through complement dependent  
4 cellular toxicity.

5 It is still a further objective of the instant invention  
6 to produce anti-cancer antibodies whose cytotoxicity is a  
7 function of their ability to catalyze hydrolysis of cellular  
8 chemical bonds.

9 Still an additional objective of the instant invention  
10 is to produce anti-cancer antibodies useful as a vaccine to  
11 produce an immune response against putative cancer antigen  
12 residing on tumor cells.

13 A further objective of the instant invention is the use  
14 of antibodies to target cell membrane proteins, such as  
15 growth receptors, cell membrane pumps and cell anchoring  
16 proteins, thereby interfering with or down regulating their  
17 function.

18 Yet an additional objective of the instant invention is  
19 the production of anti-cancer antibodies whose cell-killing  
20 utility is concomitant with their ability to effect a  
21 conformational change in cellular proteins such that a signal  
22 will be transduced to initiate cell-killing.

23 A still further objective of the instant invention is to  
24 produce anti-cancer antibodies which are useful for  
25 diagnosis, prognosis, and monitoring of cancer, e.g.

1 production of a panel of therapeutic anti-cancer antibodies  
2 to test patient samples to determine if they contain any  
3 suitable antibodies for therapeutic use.

4 Yet another objective of the instant invention is to  
5 produce novel antigens, associated with cancer processes,  
6 which can be discovered by using anti-cancer antibodies  
7 derived by the process of the instant invention. These  
8 antigens are not limited to proteins, as is generally the  
9 case with genomic data; they may also be derived from  
10 carbohydrates or lipids or combinations thereof.

11 Other objects and advantages of this invention will  
12 become apparent from the following description wherein are  
13 set forth, by way of illustration and example, certain  
14 embodiments of this invention.

#### 15 16 **Detailed Description of the Invention:**

17 It is to be understood that while a certain form of the  
18 invention is illustrated, it is not to be limited to the  
19 specific form or arrangement herein described and shown. It  
20 will be apparent to those skilled in the art that various  
21 changes may be made without departing from the scope of the  
22 invention and the invention is not to be considered limited  
23 to what is shown and described in the specification.

24 One of the potential benefits of monoclonal antibodies  
25 with respect to the treatment of cancer is their ability to

1 specifically recognize single antigens. It was thought that  
2 in some instances cancer cells possess antigens that were  
3 specific to that kind of transformed cell. It is now more  
4 frequently believed that cancer cells have few unique  
5 antigens, rather, they tend to over-express a normal antigen  
6 or express fetal antigens. Nevertheless, the use of  
7 monoclonal antibodies provided a method of delivering  
8 reproducible doses of antibodies to the patient with the  
9 expectation of better response rates than with polyclonal  
10 antibodies.

11 Traditionally, monoclonal antibodies have been made  
12 according to fundamental principles laid down by Kohler and  
13 Milstein. Mice are immunized with antigens, with or without,  
14 adjuvants. The splenocytes are harvested from the spleen for  
15 fusion with immortalized hybridoma partners. These are  
16 seeded into microtitre plates where they can secrete  
17 antibodies into the supernatant that is used for cell  
18 culture. To select from the hybridomas that have been plated  
19 for the ones that produce antibodies of interest the  
20 hybridoma supernatants are usually tested for antibody  
21 binding to antigens in an ELISA (enzyme linked immunosorbent  
22 assay) assay. The idea is that the wells that contain the  
23 hybridoma of interest will contain antibodies that will bind  
24 most avidly to the test antigen, usually the immunizing  
25 antigen. These wells are then subcloned in limiting dilution  
26 fashion to produce monoclonal hybridomas. The selection for

1 the clones of interest is repeated using an ELISA assay to  
2 test for antibody binding. Therefore, the principle that has  
3 been propagated is that in the production of monoclonal  
4 antibodies the hybridomas that produce the most avidly  
5 binding antibodies are the ones that are selected from among  
6 all the hybridomas that were initially produced. That is to  
7 say, the preferred antibody is the one with highest affinity  
8 for the antigen of interest.

9 There have been many modifications of this procedure  
10 such as using whole cells for immunization. In this method,  
11 instead of using purified antigens, entire cells are used for  
12 immunization. Another modification is the use of cellular  
13 ELISA for screening. In this method instead of using  
14 purified antigens as the target in the ELISA, fixed cells are  
15 used. In addition to ELISA tests, complement mediated  
16 cytotoxicity assays have also been used in the screening  
17 process. However, antibody-binding assays were used in  
18 conjunction with cytotoxicity tests. Thus, despite many  
19 modifications, the process of producing monoclonal antibodies  
20 relies on antibody binding to the test antigen as an  
21 endpoint.

22 Most antibodies directed against cancer cells have been  
23 produced using the traditional methods outlined above. These  
24 antibodies have been used both therapeutically and  
25 diagnostically. In general, for both these applications, the

antibody has been used as the targeting agent that delivers a payload to the site of the cancer. These antibody conjugates can either be radioactive, toxic, or serve as an intermediary for further delivery of a drug to the body, such as an enzyme or biotin. Furthermore, it was widely held, until recently, that naked antibodies had little effect *in vivo*. Both HERCEPTIN and RITUXIMAB are humanized murine monoclonal antibodies that have recently been approved for human use by the FDA. However, both these antibodies were initially made by assaying for antibody binding and their direct cytotoxicity was not the primary goal during the production of hybridomas. Any tendency for these antibodies to produce tumor cell killing is thus through chance, not by design.

Although the production of monoclonal antibodies have been carried out using whole cell immunization for various applications the screening of these hybridomas have relied on either putative or identified target antigens or on the selectivity of these hybridomas for specific tissues. It is axiomatic that the best antibodies are the ones with the highest binding constants. This concept originated from the basic biochemical principle that enzymes with the highest binding constants were the ones that were the most effective for catalyzing a reaction. This concept is applicable to receptor ligand binding where the drug molecule binding to the receptor with the greatest affinity usually has the highest probability for initiating or inhibiting a signal.



1 However, this may not always be the case since it is possible  
2 that in certain situations there may be cases where the  
3 initiation or inhibition of a signal may be mediated through  
4 non-receptor binding. The information conveyed by a  
5 conformational change induced by ligand binding can have many  
6 consequences such as a signal transduction, endocytosis,  
7 among the others. The ability to produce a conformational  
8 change in a receptor molecule may not necessarily be due to  
9 the filling of a ligand receptor pocket but may occur through  
10 the binding of another extra cellular domain or due to  
11 receptor clustering induced by a multivalent ligand.

12 The production of antibodies to produce cell killing  
13 need not be predicated upon screening of the hybridomas for  
14 the best binding antibodies. Rather, although not advocated  
15 by those who produce monoclonal antibodies, the screening of  
16 the hybridoma supernatants for cell killing or alternatively  
17 for cessation of growth of the cancerous cells may be  
18 selected as a desirable endpoint for the production of  
19 cytotoxic or cytostatic antibodies. It is well understood  
20 that the *in-vivo* antibodies mediate their function through  
21 the Fc portions and that the utility of the therapeutic  
22 antibody is determined by the functionality of the constant  
23 region or attached moieties. In this case the FAb portion of  
24 the antibody, the antigen-combining portion, will confer to  
25 the antibody its specificity and the Fc portion its

1 functionality. The antigen combining site of the antibody  
2 can be considered to be the product of a natural  
3 combinatorial library. The result of the rearrangement of the  
4 variable region of the antibody can be considered a molecular  
5 combinatorial library where the output is a peptide.  
6 Therefore, the sampling of this combinatorial library can be  
7 based on any parameter. Like sampling a natural compound  
8 library for antibiotics, it is possible to sample an antibody  
9 library for cytotoxic or cytostatic compounds.

10 The various endpoints in a screen must be differentiated  
11 from each other. For example, the difference between antibody  
12 binding to the cell is distinct from cell killing. Cell  
13 killing (cytotoxicity) is distinct from the mechanisms of  
14 cell death such as oncosis or apoptosis. There can be many  
15 processes by which cell death is achieved and some of these  
16 can lead either to oncosis or apoptosis. There is speculation  
17 that there are other cell death mechanisms other than oncosis  
18 or apoptosis but regardless of how the cell arrives at death  
19 there are some commonalities of cell death. One of these is  
20 the absence of metabolism and another is the denaturation of  
21 enzymes. In either case vital stains will fail to stain these  
22 cells. These endpoints of cell death have been long  
23 understood and predate the current understanding of the  
24 mechanisms of cell death. Furthermore, there is the  
25 distinction between cytotoxic effects where cells are killed

1 and cytostatic effects where the proliferation of cells are  
2 inhibited.

3 In a preferred embodiment of the present invention, the  
4 assay is conducted by focusing on cytotoxic activity toward  
5 cancerous cells as an end point. In a preferred embodiment,  
6 a live /dead assay kit , for example the LIVE/DEAD®  
7 Viability/Cytotoxicity Assay Kit (L-3224) by Molecular  
8 Probes, is utilized. The Molecular Probes kit provides a  
9 two-color fluorescence cell viability assay that is based on  
10 the simultaneous determination of live and dead cells with  
11 two probes that measure two recognized parameters of cell  
12 viability - intracellular esterase activity and plasma  
13 membrane integrity. The assay principles are general and  
14 applicable to most eukaryotic cell types, including adherent  
15 cells and certain tissues, but not to bacteria or yeast.  
16 This fluorescence-based method of assessing cell viability is  
17 preferred in place of such assays as trypan blue exclusion,  
18 Cr release and similar methods for determining cell viability  
19 and cytotoxicity.

20 In carrying out the assay, live cells are distinguished  
21 by the presence of ubiquitous intracellular esterase  
22 activity, determined by the enzymatic conversion of the  
23 virtually nonfluorescent cell-permeant CALCEIN AM to the  
24 intensely fluorescent Calcein. The polyanionic dye Calcein  
25 is well retained within live cells, producing an intense

uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events that do not affect these cell properties may not be accurately assessed using this method. Background fluorescence levels are inherently low with this assay technique because the dyes are virtually nonfluorescent before interacting with cells.

In addition to the various endpoints for screening, there are two other major characteristics of the screening process. The library of antibody gene products is not a random library but is the product of a biasing procedure. In the examples below, the biasing is produced by immunizing mice with fixed cells. This increases the proportion of antibodies that have the potential to bind the target antigen. Although immunization is thought of as a way to produce higher affinity antibodies (affinity maturation) in this case it is not. Rather, it can be considered as a way to shift the set of antigen combining sites towards the targets. This is also distinct from the concept of isotype switching where the functionality, as dictated by the

constant portion of the heavy chain, is altered from the initial IgM isotype to another isotype such as IgG.

The third key feature that is crucial in the screening process is the use of multitarget screening. To a certain extent specificity is related to affinity. An example of this is the situation where an antigen has very limited tissue distribution and the affinity of the antibody is a key determinant of the specificity of the antibody-the higher the affinity the more tissue specific the antibody and likewise an antibody with low affinity may bind to tissues other than the one of interest. Therefore, to address the specificity issue the antibodies are screened simultaneously against a variety of cells. In the examples below the hybridoma supernatants (representing the earliest stages of monoclonal antibody development), are tested against a number of cell lines to establish specificity as well as activity.

The antibodies are designed for therapeutic treatment of cancer in patients. Ideally the antibodies can be naked antibodies. They can also be conjugated to toxins. They can be used to target other molecules to the cancer. e.g. biotin conjugated enzymes. Radioactive compounds can also be used for conjugation.

1           The antibodies can be fragmented and rearranged  
2           molecularly. For example Fv fragments can be made; sFv-single  
3           chain Fv fragments; diabodies etc.

4           It is envisioned that these antibodies can be used for  
5           diagnosis, prognosis, and monitoring of cancer. For example  
6           the patients can have blood samples drawn for shed tumor  
7           antigens which can be detected by these antibodies in  
8           different formats such as ELISA assays, rapid test panel  
9           formats etc. The antibodies can be used to stain tumor  
10          biopsies for the purposes of diagnosis. In addition a panel  
11          of therapeutic antibodies can be used to test patient samples  
12          to determine if there are any suitable antibodies for  
13          therapeutic use.

#### 14          **Example one**

15          In order to produce monoclonal antibodies specific for a  
16          tumor sample the method of selection of the appropriate  
17          hybridoma wells is complicated by the probability of  
18          selecting wells which will produce false positive signals.  
19          That is to say that there is the likelihood of producing  
20          antibodies that can react against normal cells as well as  
21          cancer cells. To obviate this possibility one strategy is to  
22          mask the anti-normal antigen antibodies from the selection  
23          process. This can be accomplished by removing the anti-  
24          normal antibodies at the first stage of screening thereby

8  
1 revealing the presence of the desired antibodies. Subsequent  
2 limiting dilution cloning can delineate the clones that will  
3 not produce killing of control cells but will produce target  
4 cancer cell killing.

5 Biopsy specimens of breast, melanoma, and lung tumors  
6 were obtained and stored at -70°C until used. Single cell  
7 suspensions were prepared and fixed with -30°C, 70% ethanol,  
8 washed with PBS and reconstituted to an appropriate volume  
9 for injection. Balb/c mice were immunized with  $2.5 \times 10^5$ - $1 \times 10^6$   
10 cells and boosted every third week until a final pre-fusion  
11 boost was performed three days prior to the splenectomy. The  
12 hybridomas were prepared by fusing the isolated splenocytes  
13 with Sp2/0 and NS1 myeloma partners. The supernatants from  
14 the fusions were tested for subcloning of the hybridomas.  
15 Cells (including A2058 melanoma cells, CCD-12CoN fibroblasts,  
16 MCF-12A breast cells among others) were obtained from ATCC  
17 and cultured according to enclosed instructions. The HEY cell  
18 line was a gift from Dr. Inka Brockhausen. The non-cancer  
19 cells, e.g. CCD-12CoN fibroblasts and MCF-12A breast cells,  
20 were plated into 96-well microtitre plates (NUNC) 1 to 2  
21 weeks prior to screening. The cancer cells, e.g. HEY, A2058,  
22 BT 483, and HS294t, were plated two or three days prior to  
23 screening.

24 The plated normal cells were fixed prior to use. The  
25 plates were washed with 100 microliters of PBS for 10 minutes

1 at room temperature and then aspirated dry. 75 microliters  
2 of 0.01 percent glutaraldehyde diluted in PBS were added to  
3 each well for five minutes and then aspirated. The plates  
4 were washed with 100 microliters of PBS three times at room  
5 temperature. The wells were emptied and 100 microliters of  
6 one percent human serum albumin in PBS was added to each well  
7 for one hour at room temperature. The plates were then  
8 stored at four degrees Celsius.

9 Prior to the transfer of the supernatant from the  
10 hybridoma plates the fixed normal cells were washed three  
11 times with 100 microliters of PBS at room temperature. After  
12 aspiration to the microliters of the primary hybridoma  
13 culture supernatants were transferred to the fixed cell  
14 plates and incubated for two hours at 37 degrees Celsius in a  
15 8 percent CO<sub>2</sub> incubator. The hybridoma supernatants derived  
16 from melanoma was incubated with CCD-12 CoN cells and those  
17 derived from breast cancer were incubated with MCF-12a cells.

18 After incubation the absorbed supernatant was divided  
19 into two 75 microliter portions and transferred to target  
20 cancer cell plates. Prior to the transfer the cancer cell  
21 plates were washed three times with 100 microliters of PBS.  
22 The supernatant from the CCD-12 CoN cells were transferred to  
23 the A2058 and the HS294t cells, whereas the supernatant from  
24 MCF-12A cells were transferred to the HEY and BT 483 cells.  
25 The cancer cells were incubated with the hybridoma



supernatants for 18 hours at 37 degrees Celsius in an 8 percent CO<sub>2</sub> incubator.

The Live/Dead cytotoxicity assay was obtained from Molecular Probes (Eu,OR). The assays were performed according to the manufacturer's instructions with the changes outlined below. The plates with the cells were washed once with 100 microliters of PBS at 37°C. 75 to 100 microliters of supernatant from the hybridoma microtitre plates were transferred to the cell plates and incubated in a 8% CO<sub>2</sub> incubator for 18-24 hours. Then, the wells that served as the all dead control were aspirated until empty and 50 microliters of 70% ethanol was added. The plate was then emptied by inverting and blotted dry. Room temperature PBS was dispensed into each well from a multichannel squeeze bottle, tapped three times, emptied by inversion and then blotted dry. 50 microliters of the fluorescent Live/Dead dye diluted in PBS was added to each well and incubated at 37°C in a 5% CO<sub>2</sub> incubator for one hour. The plates were read in a Perkin-Elmer HTS7000 fluorescence plate reader and the data was analyzed in Microsoft Excel.

Four rounds of screening were conducted to produce single clone hybridoma cultures. For two rounds of screening the hybridoma supernatants were tested only against the cancer cells. In the last round of screening the supernatant was tested against a number of non-cancer cells as well as

1 the target cells indicated in table 1. The antibodies were  
2 isotyped using a commercial isotyping kit.

3 A number of monoclonal antibodies were produced in  
4 accordance with the method of the present invention. These  
5 antibodies, whose characteristics are summarized in Table 1,  
6 are identified as 3BD-3, 3BD-6, 3BD-8, 3BD-9, 3BD-15, 3BD-25,  
7 3BD-26 and 3BD-27. Each of the designated antibodies is  
8 produced by a hybridoma cell line deposited with the American  
9 Type Culture Collection at 10801 University Boulevard,  
10 Manassas, Va. having an ATCC Accession Number as follows:

<u>Antibody</u>	<u>ATCC Accession Number</u>
3BD-3	
3BD-6	
3BD-8	
3BD-9	
3BD-15	
3BD-25	
3BD-26	
3BD-27	

20 These antibodies are considered monoclonal after four rounds  
21 of limiting dilution cloning. The anti-melanoma antibodies  
22 did not produce significant cancer cell killing. The panel of  
23 anti-breast cancer antibodies killed 32-87% of the target  
24 cells and <1-3% of the control cells. The predominant isotype

was IgG1 even though it was expected that the majority of anti-tumor antibodies would be directed against carbohydrate antigens, and thus, be of the IgM type. There is a high therapeutic index since most antibodies spare the control cells from cell death.

Table 1. Anti-Breast Cancer Antibodies

Clones	% Cell Death			
	Target for Anti-Breast Cancer Antibodies (HEY & A2058)	Normal Fibroblast Cells (CCD-12CoN)	Fibrocystic Breast Cells (MCF-12A)	Isotype
3BD-3	74.9%	3.7%	<1%	$\gamma$ 1, $\lambda$
3BD-6	68.5%	5.6%	<1%	$\gamma$ 1, $\lambda$
3BD-8	81.9%	4.5%	2.6%	$\gamma$ 1, $\kappa$
3BD-9	77.2%	7.9%	<1%	$\gamma$ 1, $\lambda$
3BD-15	87.1%	<1 %	<1%	$\gamma$ 1, $\lambda$
3BD-26	54.8%	3.3%	<1%	$\mu$ , $\kappa$
3BD-25	32.4%	3.6%	<1 %	$\gamma$ 1, $\kappa$
3BD-27	60.1%	8.3%	1.3%	$\gamma$ 1, $\kappa$

## Example 2

In this example customized anti-cancer antibodies are produced by first obtaining samples of the patient's tumor. Usually this is from a biopsy specimen from a solid tumor or a blood sample from hematogenous tumors. The samples are prepared into single cell suspensions and fixed for injection

1 into mice. After the completion of the immunization schedule  
2 the hybridomas are produced from the splenocytes. The  
3 hybridomas are screened against a variety of cancer cell  
4 lines and normal cells in standard cytotoxicity assays. Those  
5 hybridomas that are reactive against cancer cell lines but  
6 are not reactive against normal non-transformed cells are  
7 selected for further propagation. Clones that were considered  
8 positive were ones that selectively killed the cancer cells  
9 but did not kill the non-transformed cells. The antibodies  
10 are characterized for a large number of biochemical  
11 parameters and then humanized for therapeutic use.

12 The melanoma tumor cells isolated and cell lines were  
13 cultured as described in Example 1. Balb/c mice were  
14 immunized according to the following schedule: 200,000 cells  
15 s.c. and i.p. on day 0, then 200,000 cells were injected i.p.  
16 on day 21, then 1,000,000 cells were injected on day 49, then  
17 1,250,000 cells in Freund's Complete Adjuvant were injected  
18 i.p. on day 107, and then 200,000 cells were injected on day  
19 120 i.p. and then the mice were sacrificed on day 123. The  
20 spleens were harvested and the splenocytes were divided into  
21 two aliquots for fusion with Sp2/0 (1LN) or NS-1 (2LN)  
22 myeloma partners using the methods outlined in example 1.

23 The screening was carried out 11 days after the fusion  
24 against A2058 melanoma cells and CCD-12CoN fibroblasts. Each  
25 pair of plates were washed with 100 microliters of room

temperature PBS and then aspirated to near dryness. Then 50 microliters of hybridoma supernatant was added to the same wells on each of the two plates. The spent Sp2/0 supernatant was added to the control wells at the same volume and the plates were incubated for around 18 hours at 37 degrees Celsius at a 8%CO<sub>2</sub>, 98% relative humidity incubator. Then each pair of plates were removed and in the positive control wells 50 microliters of 70% ethanol was substituted for the media for 4 seconds. The plates were then inverted and washed with room temperature PBS once and dried. Then 50uL of fluorescent live/dead dye diluted in PBS (Molecular Probes Live/Dead Kit) was added for one hour and incubated at 37 degrees Celsius. The plates were then read in a Perkin-Elmer fluorescent plate reader and the data analyzed using Microsoft Excel. The wells that were considered positive were subcloned and the same screening process was repeated 13 days later and then 33 days later. The results of the last screening is outlined in Table 2 below. A number of monoclonal antibodies were produced in accordance with the method of the present invention. These antibodies, whose characteristics are summarized in Table 2, are identified as 1LN-1, 1LN-12, 1LN-14, 2LN-21, 2LN-28, 2LN-29, 2LN-31, 2LN-33, 2LN-34 and 2LN-35. Each of the designated antibodies is produced by a hybridoma cell line deposited with the American Type Culture Collection at 10801 University Boulevard, Manassas, Va. having an ATCC Accession Number as follows:

Antibody ATCC Accession Number

1LN-1

1LN-12

1LN-14

2LN-21

2LN-28

2LN-29

2LN-31

2LN-33

2LN-34

2LN-35

Table 2, Anti-Melanoma Antibodies

Clones	% Cell Death	
	Target for Anti-Melanoma Antibodies (A2058)	Normal Fibroblast Cells (CCD-1 2CoN)
1LN-1	59.4%	<1 %
1LN-12	55.2%	1 4%
1LN-14	51.4%	<1%
2LN-21	72.0%	15.9%
2LN-28	66.6%	12.4%
2LN-29	78.2%	6.1%
2LN-31	100%	7.8%
2LN-33	94.2%	<1%

2LN-34	56.6%	11.2%
2LN-35	66.5%	6.6%

The table illustrates that clones from both the Sp2/0 and NS-1 fusions were able to produce antibodies that had a greater than 50% killing rate for cancerous cells and at the same time some of the clones were able to produce less than one percent killing of normal control fibroblasts.

The anti-cancer antibodies of the invention are useful for treating a patient with a cancerous disease when administered in admixture with a pharmaceutically acceptable adjuvant, for example normal saline, a lipid emulsion, albumen, phosphate buffered saline or the like and are administered in an amount effective to mediate treatment of said cancerous disease, for example with a range of about 1 microgram per mil to about 1 gram per mil.

The method for treating a patient suffering from a cancerous disease may further include the use of conjugated anti-cancer antibodies and would this include conjugating patient specific anti-cancer antibodies with a member selected from the group consisting of toxins, enzymes, radioactive compounds, and hematogenous cells; and administering these conjugated antibodies to the patient; wherein said anti-cancer antibodies are administered in admixture with a pharmaceutically acceptable adjuvant, for example normal saline, a lipid emulsion, albumen, phosphate

1 buffered saline or the like and are administered in an amount  
2 effective to mediate treatment of said cancerous disease, for  
3 example with a range of about 1 microgram per mil to about 1  
4 gram per mil. In a particular embodiment, the anti-cancer  
5 antibodies useful in either of the above outlined methods may  
6 be a humanized antibody.